Supplemental Figures and Legends

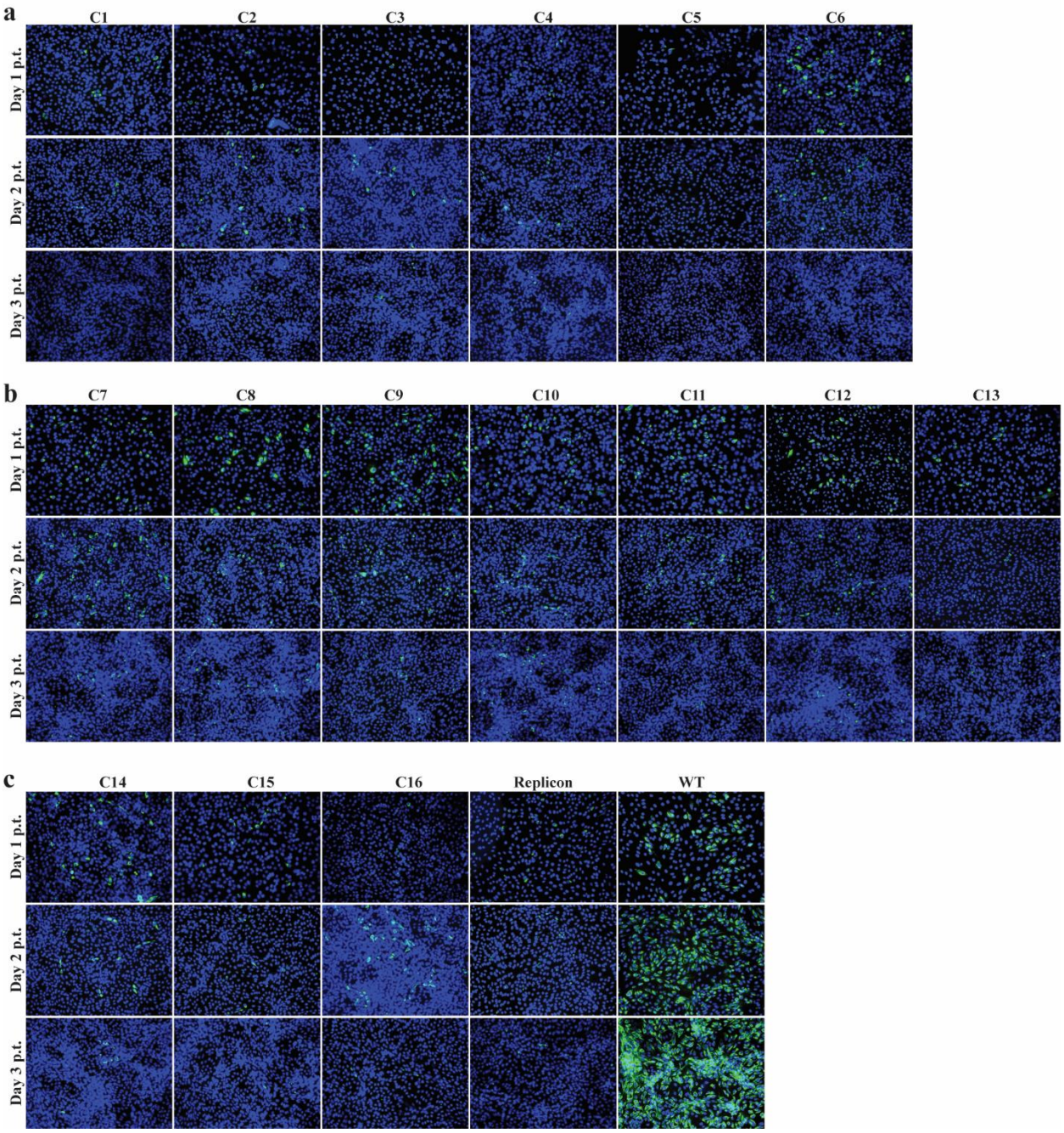


Figure S1 (Related to Figure 1). Representative IFA images for ZIKV NS3 expression in transfected cells. Vero cells were electroporated with equal amounts of C-deletion mutants or control RNAs (replicon and WT). On day 1 to 3 post-transfection, cells were fixed and stained for viral NS3 protein expression (green). Nuclei were stained with DAPI (blue). (a) Panel I mutants. (b) Panel II mutants. (c) Panel III mutants. Replicon and WT RNAs were included as controls.

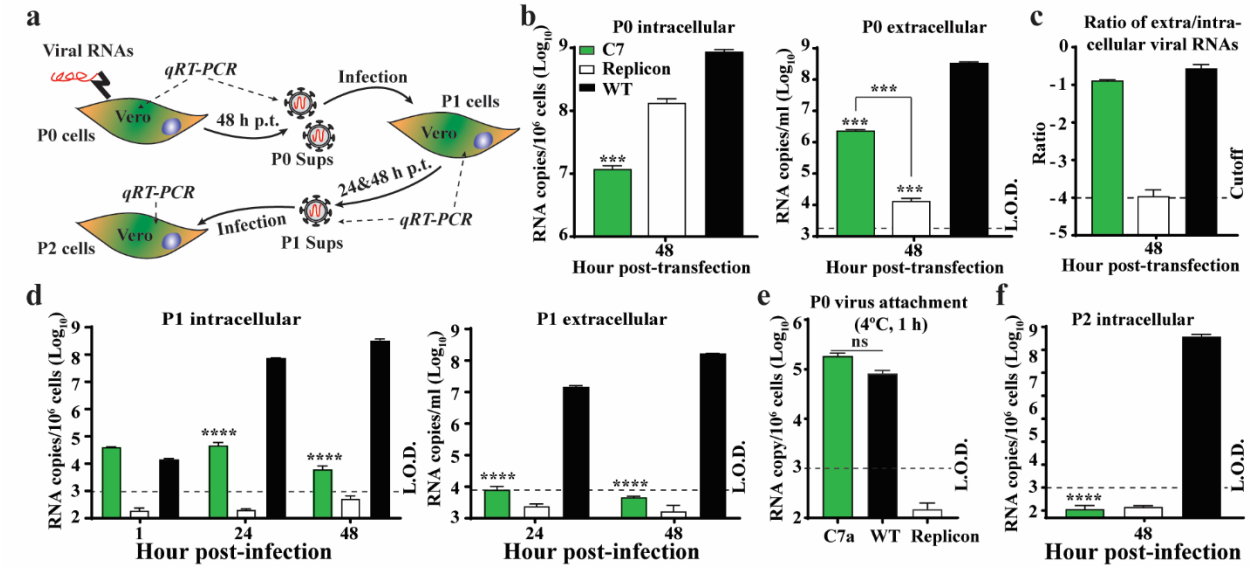


Figure S2 (Related to Figure 1). C7 mutant yields virions with a single-round infection of Vero cells. (a) Experimental design. Vero cells were electroporated with mutant C7 or control RNAs (replicon and WT). At 48 h post-transfection, intracellular and extracellular viral RNAs (P0) were quantified by real-time qRT-PCR. The supernatants (containing P0 virus) harvested from the transfected cells were used to infect naive Vero cells. At the indicated time points, intracellular and extracellular viral RNAs (P1) were measured. Culture medium (containing P1 virus) was collected at 48 h post-infection and used to infect naive Vero cells (P2). At 48 h post-infection, levels of P2 intracellular viral RNAs were determined. (b) P0 intracellular and extracellular viral RNAs. (c) Ratios of P0 extracellular/intracellular RNAs. The ratios were calculated by using total amounts of intracellular viral RNA divided by total amounts of extracellular viral RNA. (d) P1 intracellular and extracellular viral RNAs. (e) P0 virus attachment to Vero cells. After incubating Vero cells with P0 virus for 1 h at 4°C, cells were washed three times with PBS to remove unattached virions. The levels of attached virions to cells were quantified by qRT-PCR. (f) P2 intracellular viral RNAs. Means and standard deviations are shown. Statistical significances were determined using multiple t-tests.

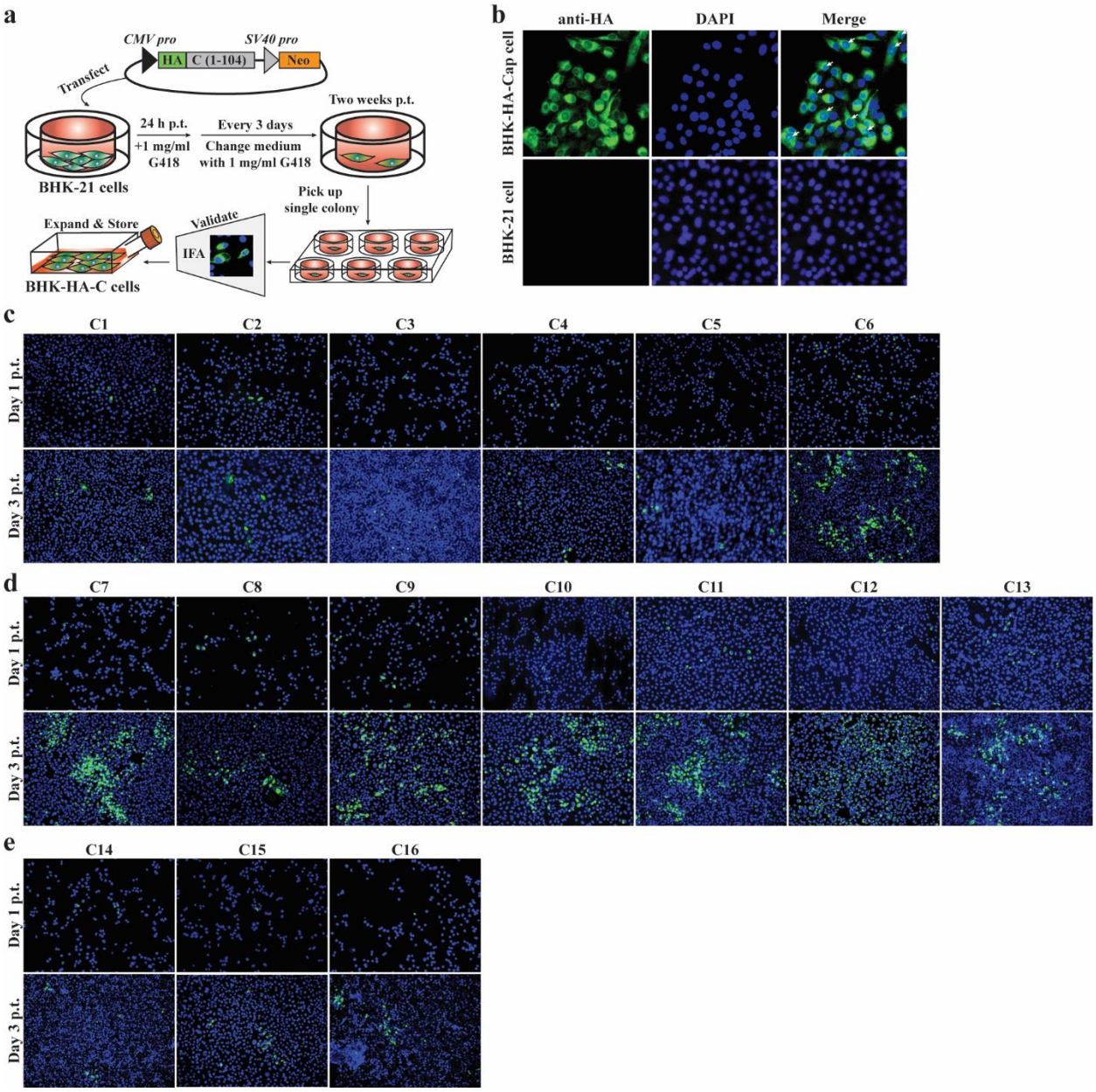


Figure S3 (Related to Figure 2). *Trans* complementation of C deletion mutants. (a) Scheme of BHK-HA-C cell line construction. BHK-21 cells were transfected with a pXJ-HA-C plasmid, which contains (i) one cassette encoding an N-terminal influenza hemagglutinin (HA) tag and ZIKV C protein (containing the complete 104 amino acids) and (ii) another cassette expressing the neomycin-resistance gene (Neo). After two weeks of selection, cell colonies were harvested and screened by IFA using HA antibodies. HA-positive cells were expanded and stored. (b) Validation of selected BHK-HA-C cell line by IFA. Cells were fixed and stained by HA antibodies to monitor HA-C protein expression (green). Arrows indicate the cells with HA-C protein found in both the

nucleus and cytoplasm. Naïve BHK-21 cells were included as a negative control. (c-e) *Trans* complementation of C deletion mutants. BHK-HA-C cells were electroporated with C-deletion mutant RNAs. At given time points, cells were fixed and stained for E protein expression (green). (c) Panel I mutants. (d) Panel II mutants. (e) Panel III mutants.

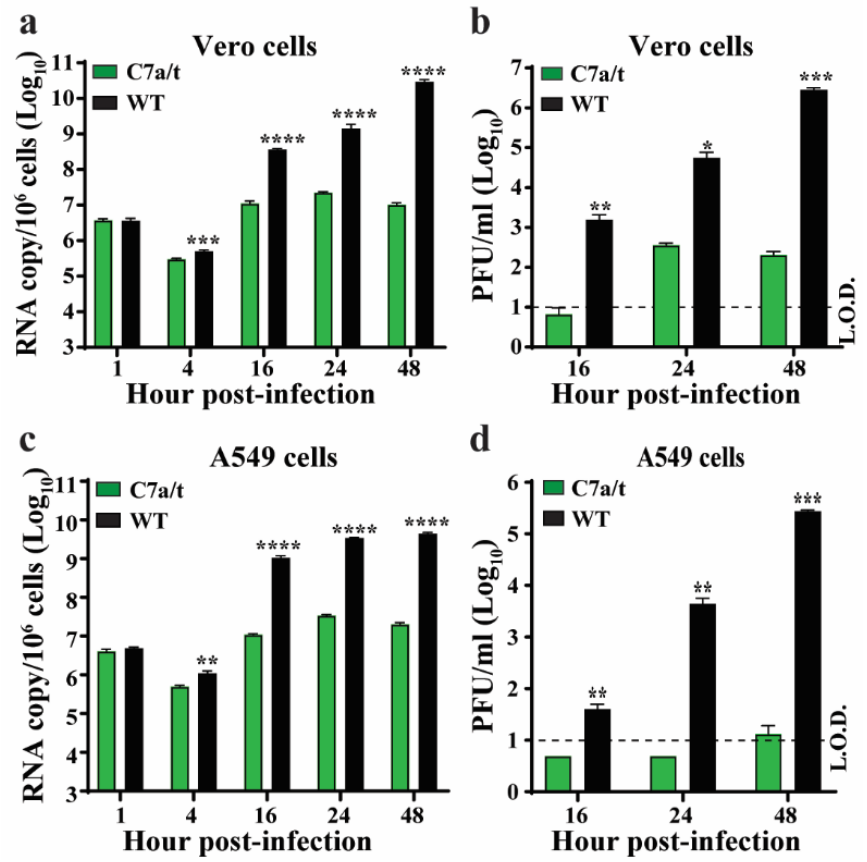


Figure S4 (Related to Figure 3). Characterization of C7a/t virus replication on Vero and A549 cells. Cells were infected with C7a/t and WT viruses at an MOI of 0.5. At the indicated time points, intracellular viral RNAs were quantified by qRT-PCR and viruses in culture medium were measured by focus-forming assay on BHK-HA-C cells. (a) Intracellular viral RNA on Vero cells. (b) Virus titers in culture medium collected from infected Vero cells. (c) Intracellular viral RNA on A549 cells. (d) Virus titers in supernatants from infected A549 cells. Means and standard deviations are shown. Statistical significances were determined using multiple t-tests.

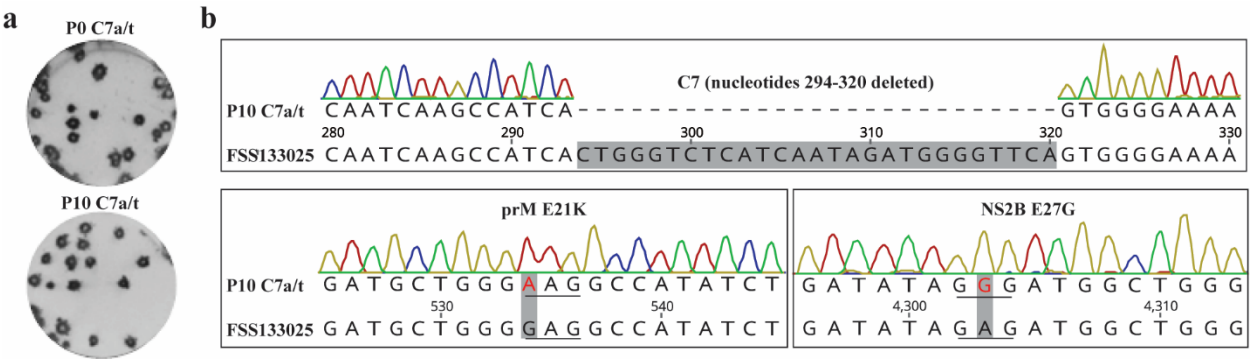


Figure S5 (Related to Figure 3). Characterization of the P10 C7a/t virus. (a) Focus morphology of P0 and P10 C7a/t viruses. (b) Representative sequence chromatograms illustrating mutations in the P10 C7a/t virus genome. The C7 deletion (top panel), prM E21K mutation (bottom left panel), and NS2B E27G mutation (bottom right panel) are indicated with reference to ZIKV FSS133025 (GenBank access number KU955593).

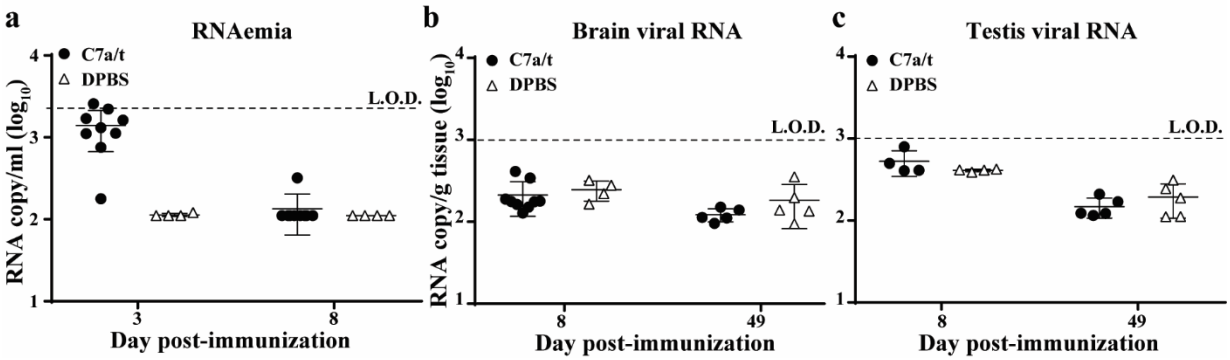


Figure S6 (Related to Figure 4). Viral RNA levels in mouse serum, brain, and testis after C7a/t virus immunization. Three-week-old A129 mice were subcutaneously injected with 10^5 FFU of C7a/t virus. At the indicated days post-immunization, viral RNA levels in serum, brain, and testis were measured by qRT-PCR. (a) Viral RNA level in serum (RNAemia). (b) Viral RNA loads in the brain. (c) Viral RNA loads in testis. Limit of detection (L.O.D.): 2,000 RNA copy/ml in serum and 1,000 copy/g tissue in the brain and testis. Means and standard deviations are shown.

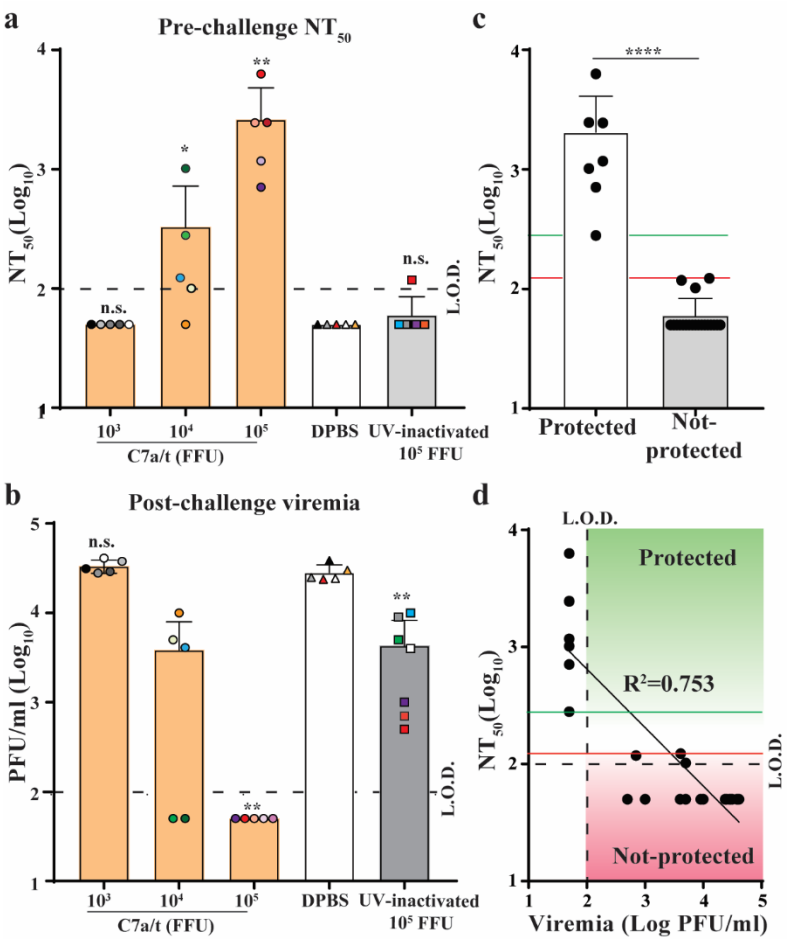
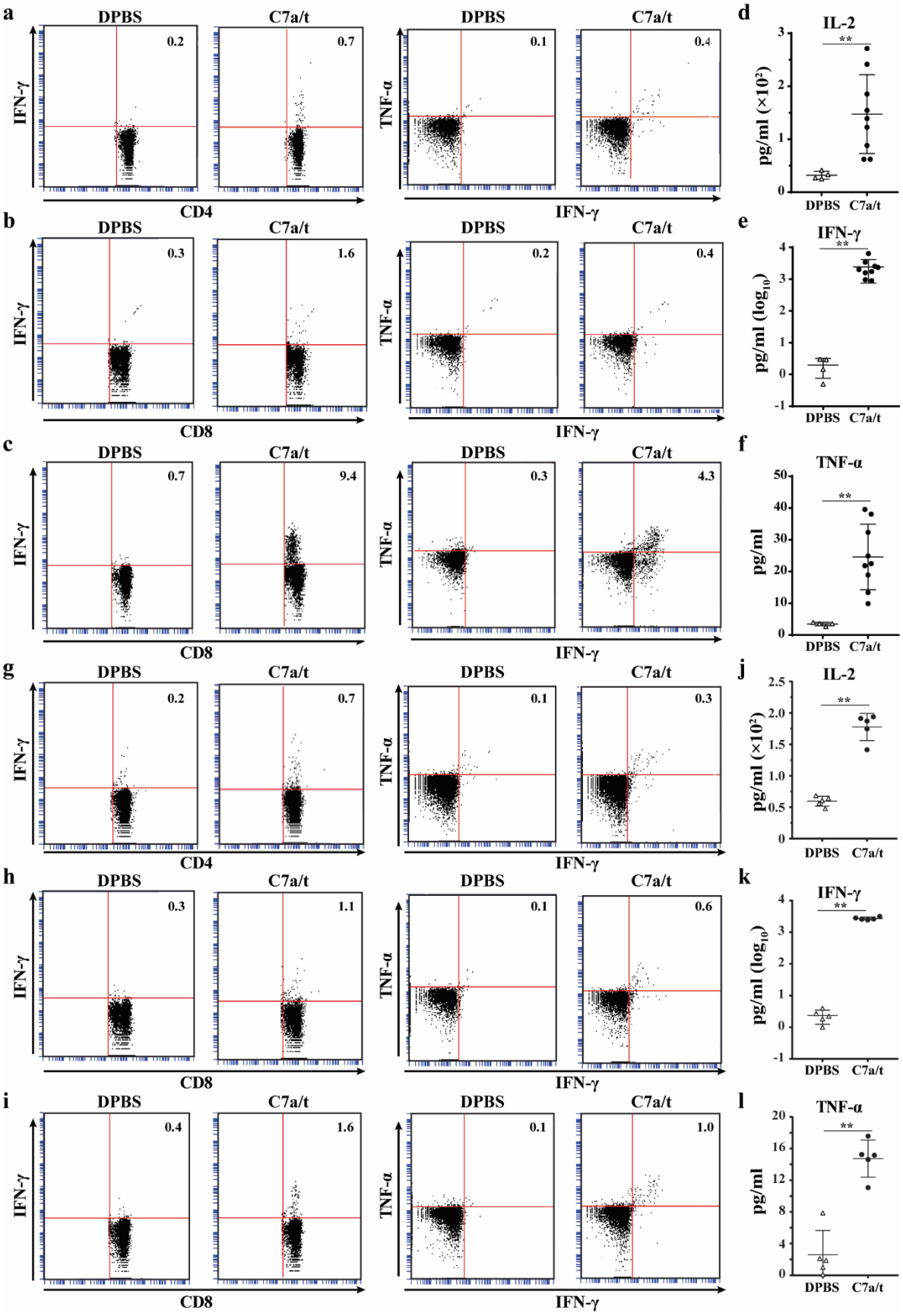


Figure S7 (Related to Figure 4). Antibody response after immunization with serial doses of C7a/t virus. (a) Pre-challenge NT_{50}s. Three-week-old mice were subcutaneously inoculated with various doses of C7a/t or UV-inactivated C7a/t viruses. On day 28 post-immunization, mice were bled to measure neutralizing antibodies, and then challenged with 10^6 PFU ZIKV strain PRVABC59. (b) Post-challenge viremia. On day 2 post-challenge, mice were bled to measure viremia using plaque assay. The unpaired nonparametric Mann-Whitney t-test was used to analyze statistical significance in viremia and NT_{50}s. Different colors in (a) and (b) represent individual animals. (c) Plot of NT_{50}s in protected and un-protected mice. The unpaired nonparametric Mann-Whitney test was used for statistical analysis. (d) Correlation between NT_{50}s and viremia. Coefficient of determination (R^2) was obtained using linear regression analysis. Geometric means and standard deviations are shown. Red and green lines represent NT_{50}s of 123 and 280, respectively. Mice were protected when $NT_{50} \geq 280$ (green zone) and un-protected when $NT_{50} \leq 123$ (red zone). Dotted lines indicate the limit of detection of assays.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DPBS
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Figure S8 (Related to Figure 6). T cell response. On day 8 or 49 post-immunization, splenocytes were isolated from C7a/t and DPBS-immunized mice, stimulated *ex vivo* with ZIKV for 24 h or an E peptide for 5 h, and stained for IFN-γ, TNF-α, and T cell markers. The cells were then gated on $CD4^+$ or $CD8^+$ T cell subsets. Representative flow cytometry images are presented. The amounts of IL-2, IFN-γ, and TNF-α in splenocyte culture were measured after stimulation with ZIKV E peptide for 3 days. (a-f) Comparison of T cell response on day 8 post-immunization in C7a/t (n=9) and DPBS (n=4)-immunized mice. (a) Percentages of IFN-γ^+ and IFN-γ^+TNF-α^+ $CD4^+$ T cells after stimulation with ZIKV. (b) Percentages of IFN-γ^+ and IFN-γ^+TNF-α^+ $CD8^+$ T cells after stimulation with ZIKV. (c) Percentages of IFN-γ^+ and IFN-γ^+TNF-α^+ $CD8^+$ T cells after stimulation with a ZIKV E peptide. Levels of IL-2 (d), IFN-γ (e) and TNF-α (f) in splenocyte cultures after stimulation with a ZIKV E peptide. Means and standard deviations are shown. (g-l) Comparison of T cell response on day 49 post-immunization in C7a/t (n=5) and DPBS (n=5)-immunized mice. (a) Percentages of IFN-γ^+ and IFN-γ^+TNF-α^+ $CD4^+$ T cells after stimulation with ZIKV. (b) Percentages of IFN-γ^+ and IFN-γ^+TNF-α^+ $CD8^+$ T cells after stimulation with ZIKV. (c) Percentages of IFN-γ^+ and IFN-γ^+TNF-α^+ $CD8^+$ T cells after stimulation with a ZIKV E peptide. Levels of IL-2 (d), IFN-γ (e), and TNF-α (f) in splenocyte culture after stimulation with a ZIKV E peptide. An unpaired nonparametric Mann-Whitney test was used for analyzing statistical significance.

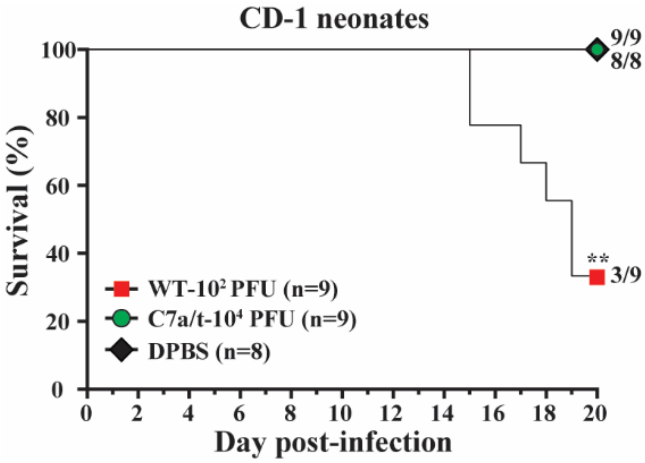

Figure S9 (Related to Figure 5). Neurovirulence analysis of C7a/t virus in neonate CD-1 mice. One-day-old CD1 mice were intracranially injected with 100 PFU of WT (n=9), 10,000 FFU of C7a/t (n=9), or DPBS (sham; n=8). Mice were monitored for morbidity and mortality over 20 days. No morbidity or mortality was observed in the DPBS or C7a/t virus-infected mice.